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Standard Analytical Protocol for *Salmonella* Typhi in Drinking Water

Office of Research and Development
National Homeland Security

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National Homeland Security Research Center

Acknowledgments

This Standard Analytical Protocol (SAP) is based on procedures evaluated by Scientific Methods, Inc. under direction of Sanjiv R. Shah at the National Homeland Security Research Center within the U.S. Environmental Protection Agency's (EPA's) Office of Research and Development for analysis of *Salmonella enterica* subsp. *enterica* serotype Typhi (*Salmonella Typhi*) in drinking water samples. Technical support and data evaluation were provided by Computer Sciences Corporation under EPA Contract No. EP-C-05-045.

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The procedures described in this document are intended for use in laboratories when analyzing water samples in support of remediation efforts following a homeland security incident. The culture-based procedures provide viability determination, identification, and either qualitative or quantitative results. The sample preparation procedures are deemed the most appropriate for the wide variety of water matrices to be examined. To the extent possible, these procedures were developed to be consistent with other federal agency procedures. These procedures do not include the sample collection, rapid screening, field techniques, or molecular techniques that may accompany laboratory analysis.

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Foreword

The mission of the U.S. Environmental Protection Agency (EPA) is to protect human health and to safeguard the natural environment – the air, water, and land upon which life depends. After the 2001 terrorist attacks including the anthrax bioterrorism event, the EPA's mission was expanded to address critical needs related to homeland security. Presidential directives identified EPA as the primary federal agency responsible for the protection and decontamination of indoor-outdoor structures and water infrastructure vulnerable to chemical, biological, or radiological (CBR) terror attacks.

The National Homeland Security Research Center (NHSRC) within the Office of Research and Development (ORD) is EPA's focal point for providing expertise, and for conducting and reporting research to meet its homeland security mission needs. One specific focus area of the NHSRC's research is to support the Environmental Response Laboratory Network (ERLN), a nationwide association of federal, state, local, and commercial environmental laboratories, established by EPA. The ERLN can be deployed in response to a large-scale environmental disaster to provide consistent analytical capabilities, capacities, and quality data in a systematic and coordinated manner. To this end, the NHSRC has worked with experts across EPA and other federal agencies to develop standard analytical protocols (SAPs) to be used in support of the response to national homeland security related incidents.

This Standard Analytical Protocol (SAP) is for the identification, confirmation, and quantitation of *Salmonella* Typhi in drinking water samples, using selective and non-selective media followed by biochemical characterization and serological confirmation.

NHSRC has made this publication available to assist in preparing for and recovering from disasters involving *Salmonella* Typhi contamination. This work specifically represents an important step in EPA's support for the ERLN and moves the agency closer to achieving its mission to support homeland security and its overall mission to protect human health and the environment.

Gregory D. Sayles, Ph.D., Acting Director

National Homeland Security Research Center

Office of Research and Development

U.S. Environmental Protection Agency

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1.0
Scope and Application

1.1
This Standard Analytical Protocol (SAP) is for the identification, confirmation, and quantitation of *Salmonella enterica* subspecies *enterica* serotype Typhi (referred to as "*S.* Typhi" in this document) in drinking water samples, using selective and non-selective media followed by biochemical and serological confirmation.

1.2
This method has been adapted from the American Society for Microbiology's *Manual of Clinical* Microbiology (Reference 15.1) and the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (Reference 15.2) for use by laboratories when analyzing samples in support of U.S. Environmental Protection Agency (EPA) homeland security efforts.

1.3
S. Typhi is the causative agent of typhoid fever. Due to the infectious nature of the bacterium and the potential for transmission to humans, all procedures should be performed in laboratories that use, at a minimum, biological safety level (BSL)-2. Use of a biological safety cabinet is recommended for any aerosol-generating procedures (Reference 15.3).

1.4
All sample handling, analysis, and reporting of results must be performed in accordance with guidelines established in the SAP. Laboratories must have requisite resources in place prior to use of these procedures.

1.5
This method is not intended for analysis of microorganisms other than *S.* Typhi in drinking water samples.

2.0
Summary of Method

2.1
S. Typhi can be identified in drinking water samples using selective media and biochemical and serological analyses. Bacterial densities can be estimated using the most probable number (MPN) approach (see Section 11.0).

2.2
For qualitative results, samples are diluted 1:1 in double-strength universal pre-enrichment (UP) broth. Samples are incubated at 35.0°C ± 0.5°C for 24 ± 2 hours.

2.3
For quantitative results, samples are analyzed as received. Samples are analyzed using a 15-tube MPN. Inoculated UP broth tubes are incubated at 35.0°C ± 0.5°C for 24 ± 2 hours.

2.4
One mL of each UP broth culture (MPN and qualitative analyses tubes) with positive growth (turbidity) is transferred to selenite cystine broth (SCB). Tubes are incubated at 35.0°C ± 0.5°C for 18 ± 2 hours. Tubes with growth are streaked onto bismuth sulfite (BS) and Miller-Mallinson (MM) agars. Plates are incubated at 35.0°C ± 0.5°C for 24 – 48 hours.

2.5
Isolated, typical colonies are sub-cultured onto tryptic soy agar (TSA) and submitted to serological and biochemical confirmation. Serological typing is by agglutination using Vi antiserum, followed by biochemical characterization using commercially available test strips (e.g., API 20E® or equivalent) or with a group of selected individual biochemical tests.

2.6
UP broth tubes (MPN and qualitative analysis) exhibiting growth (turbidity) or growth from agar plates (BS or MM), may be confirmed by real-time polymerase chain reaction (PCR) in place of serological and biochemical confirmation.

2.7
Quantitation of *S.* Typhi is determined using the MPN technique (Flowchart 16.1). Tubes that are confirmed positive for *S.* Typhi are used to determine MPN.

3.0
Acronyms and Abbreviations

ATCC®	American Type Culture Collection
BS	Bismuth sulfite
BSL	Biological safety level
°C	Degrees Celsius
DHB	2,3-dihydroxybenzoate
EPA	U.S. Environmental Protection Agency
g	Gram(s)
L	Liter(s)
µL	Microliter
mL	Milliliter(s)
mm	Millimeter(s)
MM	Miller-Mallinson
MPN	Most probable number
N	Normal – one equivalent weight per liter
NA	Not applicable
NIST	National Institute of Standards and Technology
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPE	Personal protective equipment
psi	Pounds per square inch
SAP	Standard Analytical Protocol
SCB	Selenite cystine broth
TSA	Tryptic soy agar
UP	Universal pre-enrichment
w/v	Weight to volume ratio

4.0
Interferences and Contamination

4.1
Low recoveries of *S.* Typhi may be caused by the presence of high numbers of competing or inhibitory organisms (e.g., other Enterobacteriaceae), or toxic substances (e.g., metals or organic compounds).

4.2
A viable but non-culturable state of *S.* Typhi may also account for low recoveries (Reference 15.4).

5.0
Safety

5.1 Laboratory Hazards

To prevent transmission, disposable gloves should be worn when working with this organism. Hands should be washed immediately following removal of gloves. Direct and indirect contact of intact or broken skin with cultures and/or contaminated laboratory surfaces and accidental parenteral inoculation, are the primary hazards to laboratory personnel. Rarely, exposure to infectious aerosols may occur. Staff should apply safety procedures used for pathogens when handling all samples.

5.2 Recommended Precautions

5.2.1

S. Typhi is a BSL-2 pathogen and all procedures should be performed in laboratories that use, at a minimum, BSL-2 practices (Reference 15.3). This includes prohibiting eating, drinking, smoking, handling contact lenses, applying cosmetics, and storing food and drink in the laboratory.

5.2.2

A Class II biological safety cabinet is recommended for sample manipulations where the risk of aerosol production is high. Production of aerosols should be avoided.

5.2.3

Disposable materials are recommended for sample manipulation.

5.2.4

Mouth-pipetting is prohibited.

5.2.5

The analyst must know and observe normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of media, cultures, reagents, and materials, including operation of sterilization equipment.

5.2.6

Personal Protective Equipment (PPE)

5.2.6.1

Disposable nitrile gloves should be worn at all times to prevent contact with infectious materials. Gloves should be changed whenever they are visibly soiled. Aseptic technique should be used when removing gloves and other protective clothing.

5.2.6.2

Protective goggles and/or non-breakable, chemical-resistant glasses should be worn, as appropriate.

5.2.6.3

Laboratory coats covering arms and clothing, and closed in the front, should be worn at all times. Laboratory coats that become soiled should be changed.

5.2.7

This protocol does not address all safety issues associated with its use. Please refer to *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition (Reference 15.3) for additional safety information. A reference file of Material Safety Data Sheets should be available to all personnel involved in analyses.

6.0
Equipment and Supplies

6.1
Autoclave or steam sterilizer, capable of achieving 121°C (15 pounds per square inch [psi]) for 15 minutes

6.2
Autoclave bags, aluminum foil, or kraft paper

6.3
Balance, top loading, with ASTM International Class S reference weights, capable of weighing 100 g ± 0.1g

6.4
Beakers, glass or plastic (assorted sizes)

6.5
Biological safety cabinet, Class II (optional)

6.6
Borosilicate glass or plastic screw-cap, wide-mouth bottles, sterile (e.g., 250 mL)

6.7
Borosilicate glass culture tubes, with autoclavable screw or snap caps (25 × 150 mm)

6.8
Borosilicate glass culture tubes, with autoclavable screw or snap caps (16 × 150 mm)

6.9
Erlenmeyer flasks (500 mL, 1 L, 2 L)

6.10
Graduated cylinders (assorted sizes)

6.11
Gloves, sterile, nitrile, or equivalent

6.12
Incubators, microbiological type, maintained at 35.0°C ± 0.5°C

6.13
Inoculation loops, sterile, disposable

6.14
Parafilm® or equivalent

6.15
Petri dishes, sterile, plastic (15 × 100 mm)

6.16
pH meter

6.17
Pipettes, standard tip, sterile, plastic, disposable (assorted sizes)

6.18
Pipetting device (automatic or equivalent)

6.19
Stirring hotplates and stir bars

6.20
Test tube racks

6.21
Thermometer, National Institute of Standards and Technology (NIST)-traceable

6.22
Tissues, lint-free (Kimwipes® or equivalent)

6.23
Weigh paper and boats

7.0
Reagents and Standards

7.1
Reagent-grade chemicals must be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 15.5). For suggestions regarding the testing of reagents not listed by the American Chemical Society, see *AnalaR Standards for Laboratory Chemicals* (Reference 15.6) and *United States Pharmacopeia and National Formulary 24* (Reference 15.7).

7.2
Whenever possible, use commercially available culture media. The agar used in the preparation of culture media must be microbiological grade. Storage requirements for prepared media and reagents are provided in Table 2 (Section 7.16).

7.3
Reagent-grade water must conform to specifications in *Standard Methods for the Examination of Water and Wastewater*, 21st Edition, Section 9020 (Reference 15.8).

7.4
If *S.* Typhi CVD 909 (non-virulent vaccine strain) is used as the positive control, all media must be supplemented with 2,3-dihydroxybenzoate (DHB) to obtain appropriate growth.

7.5 Phosphate Buffered Saline (PBS)
Prepare reagent according to the procedure below.

7.5.1 Composition:

Monosodium phosphate (NaH_2PO_4)	0.58 g
Disodium phosphate (Na_2HPO_4)	2.50 g
Sodium chloride	8.50 g
Reagent-grade water	1.0 L

7.5.2
Dissolve reagents in 1 L reagent-grade water, adjust pH to 7.4 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, and dispense appropriate volumes in screw-cap bottles or tubes and autoclave at 121°C (15 psi) for 15 minutes.

7.6 Universal Pre-enrichment (UP) Broth
Commercially prepared media is recommended. Dehydrated medium (Difco™ 223510 or equivalent) may be used. If commercially prepared media are not available, prepare 1X, 2X, and 3X UP broth according to the following.

7.6.1 Composition:

	1X	2X	3X
Pancreatic digest of casein	5.0 g	10.0 g	15.0 g
Proteose peptone	5.0 g	10.0 g	15.0 g
Monopotassium phosphate	15.0 g	30.0 g	45.0 g
Disodium phosphate	7.0 g	14.0 g	21.0 g
Sodium chloride	5.0 g	10.0 g	15.0 g
Dextrose	0.5 g	1.0 g	1.5 g
Magnesium sulfate	0.25 g	0.5 g	0.75 g
Ferric ammonium citrate	0.1 g	0.2 g	0.3 g
Sodium pyruvate	0.2 g	0.4 g	0.6 g
Reagent-grade water	1.0 L	1.0 L	1.0 L

7.6.2

Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar. Adjust pH to 6.3 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring to 1 L. For 1X UP broth, aseptically dispense 10 mL volumes into 25 × 150 mm tubes. For 2X UP broth, dispense in appropriate volumes (e.g., 100 mL). For 3X UP broth, aseptically dispense 5 and 10 mL volumes into 25 × 150 mm tubes. Autoclave at 121°C (15 psi) for 15 minutes.

7.7 Selenite Cystine Broth (SCB)

Commercially prepared media is recommended. Dehydrated medium (Difco™ 268740 or equivalent) may be used. If commercially prepared media are not available, prepare media using procedures in Sections 7.7.1 and 7.7.2.

7.7.1 Composition:

Pancreatic digest of casein	5.0 g
Lactose	4.0 g
Sodium phosphate	10.0 g
Sodium selenite	4.0 g
L-Cystine	0.01 g
Reagent-grade water	1.0 L

7.7.2

Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar. Adjust pH to 7.0 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring to 1 L. Boil for one minute with rapid stir bar agitation to dissolve completely. DO NOT AUTOCLAVE. Aseptically dispense 10 mL volumes into 16 × 150 mm tubes. Use medium within 48 hours of preparation.

Note: *A brick-red precipitate may appear if the medium is overheated during preparation or exposed to excessive moisture during storage. If this occurs, the medium should be discarded and a new batch prepared.*

7.8 Bismuth Sulfite (BS) Agar

Commercially prepared media is recommended. Dehydrated medium (Difco™ 273300 or equivalent) may be used. If commercially prepared media are not available, prepare medium using procedures in Sections 7.8.1 and 7.8.2.

7.8.1 Composition:

Beef extract	5.0 g
Peptone	10.0 g
Dextrose	5.0 g
Disodium phosphate	4.0 g
Ferrous sulfate	0.3 g
Bismuth sulfite indicator	8.0 g
Brilliant green	0.025 g
Agar	20.0 g
Reagent-grade water	1.0 L

7.8.2

Add reagents to 1 L of reagent-grade water and mix thoroughly using a stir bar and hot plate. Boil for one minute with rapid stir bar agitation to dissolve completely. DO NOT AUTOCLAVE. The pH should be 7.7 ± 0.2. Aseptically pour 12 – 15 mL into each 15 × 100 mm sterile Petri plate. Use plates within 48 hours of preparation.

7.9 Miller-Mallinson (MM) Agar

Commercially prepared plates (e.g., Northeast Laboratory Services or equivalent) should be used.

Note: *Formulation is provided only to ensure that the appropriate medium is used for analyses; laboratories should use commercially prepared plates.*

7.9.1 Composition:

Sodium thiosulfate	6.8 g
a-Lactose	10.0 g
D(+) Cellobiose	5.0 g
Dipeptone	3.5 g
Yeast extract	3.0 g
Sodium chloride	3.0 g
Trizma® HCl	2.3 g
Beef extract	2.0 g
D(+) Trehalose dehydrate	1.33 g
D-Mannitol	1.2 g
Ferric ammonium citrate	0.8 g
Trizma® base	0.7 g
X-Gal	0.1 g
Agar	15.0 g
Tergitol™ (Niaproof®)	4.5 mL
Reagent-grade water	1.0 L

7.10 Tryptic Soy Agar (TSA)

Commercially prepared media is recommended. Dehydrated medium (Difco™ 236950 or equivalent) may be used. If commercially prepared media are not available, prepare media using procedures in Sections 7.10.1 and 7.10.2.

7.10.1 Composition:

Pancreatic digest of casein	15.0 g
Enzymatic digest of soybean meal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.10.2

Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Heat to dissolve completely. Adjust pH to 7.3 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring up to 1 L. Autoclave at 121°C (15 psi) for 15 minutes. Aseptically pour 12 – 15 mL into each 15 × 100 mm sterile Petri plate.

7.11

Saline, physiological (0.85% w/v): Dissolve 0.85 g NaCl in 100 mL of reagent-grade water. Autoclave at 121°C (15 psi) for 15 minutes. Cool to room temperature.

7.12 *Salmonella* Vi antiserum (BD™ 228271 or equivalent)

7.13 Biochemical test strip (bioMérieux API 20E® or equivalent)

7.14 Oxidase reagent (BD™ DrySlide™ 231746 or equivalent)

7.15

Positive and negative control cultures that are to be used with this protocol are listed in Table 2. Use of these controls is discussed in Section 9.0.

Table 1. Positive and Negative Control Cultures for Described Tests

Media/Tests	Positive Control [1], [2]	Negative Control
SCB	*S.* Typhi CVD 909	*Enterococcus faecalis* (ATCC® 29212™)
BS	*S.* Typhi CVD 909	*Enterococcus faecalis* (ATCC® 29212™)
MM	*S.* Typhi CVD 909	*Enterococcus faecalis* (ATCC® 29212™)
Vi serum agglutination	*S.* Typhi CVD 909	*Enterococcus faecalis* (ATCC® 29212™)
Biochemical test strip	*S.* Typhi CVD 909	*Pseudomonas aeruginosa* (ATCC® 27853™)
Oxidase	*Pseudomonas aeruginosa* (ATCC® 27853™)	*S.* Typhi CVD 909

[1] *S.* Typhi CVD 909 is a non-virulent vaccine strain; if used, all media must be supplemented with DHB to obtain appropriate growth.

[2] Other strains of *S.* Typhi may be used in place of CVD 909.

BS – Bismuth sulfite MM – Miller-Mallinson SCB – Selenite cystine broth

7.16

Storage temperatures and times for prepared media and reagents are provided in Table 2 Follow manufacturers' guidelines for storage and expiration of all commercially prepared reagents.

Table 2. Storage Temperatures and Times for Prepared Media and Reagents [1]

Media/Reagents	Storage Temperature	Storage Time
PBS, saline (in screw-cap containers)	<10°C and above freezing	3 months
Tubes: UP broth	<10°C and above freezing	2 weeks in loose-cap tubes 3 weeks in screw-cap tubes
Plates: TSA, MM	<10°C and above freezing	2 weeks
Plates: BS Tubes: SCB	<10°C and above freezing	48 hours

[1] If media/reagent is refrigerated, remove from refrigerator 1–1.5 hours prior to inoculation to ensure that it reaches room temperature prior to use.

8.0
Calibration and Standardization

8.1
Check temperature in incubators twice daily, a minimum of four hours apart, to ensure operation is within stated limits of the method. Record daily measurements in an incubator log book.

8.2
Check temperature in refrigerators/freezers at least once daily to ensure operation is within stated limits of the method. Record daily measurements in a refrigerator/freezer log book.

8.3
Calibrate thermometers and incubators semi-annually against a NIST-certified thermometer or against a thermometer that meets the requirements of NIST Monograph SP 250-23 (Reference 15.9). Check mercury columns for breaks.

8.4
Calibrate pH meter prior to each use with two of three standards (e.g., pH 4.0, 7.0, or 10.0) closest to the range being tested.

8.5
Calibrate analytical and top-loading balances with ASTM International Class S reference weights once per month, at a minimum. Check each day prior to use with Class S weights.

8.6
Re-certify biological safety cabinets once per year. Re-certification must be performed by a qualified technician.

9.0
Quality Control

9.1 General

Each laboratory that uses this method is required to operate a formal quality assurance program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. Specific quality control procedures for use with this method are discussed below.

Note: *Following testing and validation, this method may be updated to include quality control criteria for initial and ongoing demonstration of capability as well as matrix spike/matrix spike duplicates.*

9.2 Negative Controls

9.2.1

The laboratory should analyze negative controls to ensure that all media and reagents are performing properly. Negative controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should analyze a negative control every day that samples are analyzed. Recommended negative control organisms are provided in Table 1 (Section 7.16), and descriptions of negative results are provided in Table 3 (Section 10.8).

9.2.2

Analysis of negative controls is conducted by inoculating media and performing biochemical and serological analyses with known negative control organisms as described in Section 10.0. The negative control is treated as a sample and submitted to the same analytical procedures.

9.2.3

If a negative control fails to exhibit the appropriate response, check and/or replace the associated media, reagents, and/or negative control organism, and re-analyze the appropriate negative control and corresponding sample(s).

9.2.4

Viability of the negative controls should be demonstrated on a monthly basis, at a minimum, using a non-selective media (e.g., TSA).

9.3 Positive Controls

9.3.1

The laboratory should analyze positive controls to ensure that all media and reagents are performing properly. Positive controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should analyze a positive control every day that samples are analyzed. Recommended positive control organisms are provided in (Section 7.16), and descriptions of positive results are provided in Table 3 (Section 10.8).

9.3.2

Analysis of positive controls is conducted by inoculating media and performing biochemical and serological analyses with known positive organisms as described in Section 10.0. The positive control is treated as a sample and submitted to the same analytical procedures.

9.3.3

If a positive control fails to exhibit the appropriate response, invalidate the sample results; check and/or replace the associated media, reagents, and/or the positive control organism, and re-analyze the appropriate positive control and corresponding sample(s).

9.4 Method Blank

On an ongoing basis, the laboratory should perform a method blank every day that samples are analyzed using sterile PBS (Section 7.5) to verify the sterility of equipment, materials, and supplies. The method blank is treated as a sample and submitted to the same analytical procedures. Absence of growth indicates freedom from contamination by the target organisms.

9.5 Media Sterility Check

Test sterility of PBS and media (UP broth, SCB, BS, MM, TSA) by incubating one unit (tube or plate) either from each batch at 35.0°C ± 0.5°C for 24 ± 2 hours or 48 ± 3 hours, as appropriate. Absence of growth indicates the media are sterile. On an ongoing basis, a media sterility check should be done every day that samples are analyzed.

10.0
Procedures

Process samples promptly upon receipt. *S.* Typhi is a pathogen, and all samples should be handled with caution, using appropriate BSL-2 procedures and PPE. A Class II biological safety cabinet is recommended for sample manipulations where the risk of aerosol production is high.

10.1 Qualitative Sample Analysis

Add a sample volume (e.g., 100 mL) to an equal volume of double-strength UP broth (Section 7.6). Incubate at 35.0°C ± 0.5°C for 24 ± 2 hours. After incubation, proceed to Section 10.3 for selective enrichment of *S.* Typhi.

10.2 Quantitative Sample Analyses

A multiple-tube assay incorporating differential sample volumes is used to estimate *S.* Typhi densities in undiluted or diluted samples. If low levels of *S.* Typhi are suspected, larger sample volumes (20.0 mL of original sample) should be used to inoculate the first row of tubes in the series. If high levels of *S.* Typhi are suspected, additional serial dilutions should be used. See Flowchart 16.1 for an overview of the sample dilution and inoculation scheme. A minimum sample volume of 156 mL is required if 20 mL volumes are used to inoculate the first row of tubes.

10.2.1 Sample inoculation

Arrange UP broth tubes in three rows (10 mL of 3X, 5 mL of 3X, and 10 mL of 1X) of five tubes each. Inoculate the first row of tubes (10 mL of 3X UP broth) with 20 mL of the undiluted sample. Inoculate 10 mL of the undiluted sample into each of the tubes in the second row (5 mL of 3X UP broth). Inoculate 1 mL from the initial sample into each of the tubes in the third row (10 mL of 1 UP broth). See Flowchart 16.1 for an overview of the sample inoculation scheme.

10.2.2 Sample dilutions

Samples may require serial dilution prior to inoculation due to high levels of *S.* Typhi. If analyzing serially diluted samples, 1.0 mL of each dilution will be used to inoculate each tube of 1X UP broth, as appropriate.

10.2.3 Growth

Incubate tubes at 35.0°C ± 0.5°C for 24 ± 2 hours. Proceed to Section 10.3 for selective enrichment of *S.* Typhi.

10.3 Selenite Cystine Broth Culture

10.3.1

For each tube with growth, gently swirl the tube to mix and transfer 1.0 mL to a set of tubes with 10 mL of SCB. Incubate at 35.0°C ± 0.5°C for 18± 2 hours.

10.3.2

Proceed to Section 10.4 for isolation on BS and MM agars.

10.4 Isolation on Bismuth Sulfite and Miller-Mallinson Agar Plates

See Flowchart 16.2 for an overview of the colony identification procedures.

10.4.1

Select all SCB tubes with growth (qualitative and quantitative) and streak for isolation onto BS and MM plates using a sterile inoculation loop (20 µL).

10.4.2

Incubate plates for 24 – 48 hours at 35.0°C ± 0.5°C. Typical *S.* Typhi colonies are green-black with metallic sheen on BS agar and black on MM agar.

Note: *It is important to streak for isolation. In areas of heavy growth on BS agar, S. Typhi colonies appear light green and may be misinterpreted as negative for S. Typhi.*

10.5 Isolation on Tryptic Soy Agar Plates

10.5.1

Examine plates at 24 ± 2 hours. For each BS and MM plate with typical colonies, streak a single typical colony onto TSA and incubate at 35.0°C ± 0.5°C. If no typical colonies are observed, continue incubation at 35.0°C ± 0.5°C for a total of 48 ± 3 hours. Re-examine plates, streak typical colonies onto TSA, and incubate at 35.0°C ± 0.5°C.

10.5.2

Seal the BS and MM plates with Parafilm® and store at <10°C and above freezing for use as backup plates. Use the TSA plates for serological and biochemical analyses.

10.6 Biochemical Analyses

10.6.1 Oxidase Test (BD™ DrySlide™ 231746 or equivalent)

Following manufacturer's instructions, transfer a small amount of growth from an isolated colony to the slide. Oxidase-positive bacteria turn the reagent dark purple within 20 seconds. *S.* Typhi is oxidase-negative. Results should be compared with those for positive and negative controls (Table 1) analyzed at the same time.

10.6.2 Biochemical Test Strips (API 20E® or equivalent)

Emulsify the remainder of the colony in sterile physiological saline (Section 7.11). Follow manufacturer's instructions to inoculate wells and add appropriate reagents. Incubate test strip according to manufacturer's instructions. Add additional reagents, read, and record results.

10.6.3 Alternative Biochemical Tests

The following individual biochemical tests may be used instead of biochemical test strips to identify *S.* Typhi:

- Citrate
- Glucose fermentation
- H_2S
- Indole
- Lysine
- Motility
- Ornithine
- Urease
- Voges-Proskauer

S. Typhi ferments glucose without the production of gas; produces trace amounts of H_2S, is lysine decarboxylase positive, motile and produces negative reactions for citrate, indole, ornithine, urease, and Voges-Prokauer.

10.7 Serological Analyses

Use a single, isolated, large colony (2 – 3 mm diameter) from each TSA plate for serological and biochemical test strip and oxidase analyses.

10.7.1

Take a portion of growth from a typical colony from each of the TSA plates and emulsify growth using sterile physiological saline (Section 7.11). Place two discrete drops of emulsified growth onto a slide. To the first drop of emulsified growth, add one drop of *Salmonella* Vi antiserum (Section 7.12). To the second drop of emulsified growth, add one drop of sterile saline (as a visual comparison).

10.7.2

Observe under magnification for an agglutination reaction, which indicates a positive result. *S.* Typhi is agglutination-positive for Vi antiserum. Results should be compared with those for positive and negative controls (Table 1, Section 7.15) analyzed at the same time.

10.8 Description of Control and *Salmonella* Typhi Results

Typical results are provided in Table 3.

Table 3. Positive and Negative Result Descriptions and ***Salmonella*** Typhi Results

Medium/Test	***S.* Typhi Results**[(1)]	**Positive Control Result and Description**	**Negative Control Result and Description**
SCB	Positive	Growth	No growth
BS	Positive	Green-black colonies with metallic sheen	No growth
MM	Positive	Black colonies	No growth
Oxidase	Negative	Purple to violet color change within 20 seconds	Colorless or very light pink color change over time
Vi antiserum [1]	Positive	Agglutination	No agglutination
Biochemical test strip	Consult manufacturer's instructions		

[(1)] Most wild type *S.* Typhi strains will be Vi antiserum positive.

BS – Bismuth sulfite SCB – Selenite cystine broth

MM – Miller-Mallinson

11.0 Data Analysis and Bacterial Density Calculation

11.1 Most Probable Number (MPN) Technique

Estimation of bacterial densities may be determined based on the number of tubes positive for *S.* Typhi either by morphological, biochemical, and serological results or PCR.

11.2 Calculation of MPN

If only three rows of tubes were analyzed, identify appropriate MPN value using either Table 5 or 6, depending on volumes assayed. If more than three rows of tubes were analyzed, the appropriate rows must be selected and MPN value calculated as described in Sections 11.2.1 and 11.2.2. Table 5 should only be used for volumes of 20.0 mL, 10.0 mL, and 1.0 mL. To select MPN values for volumes of 10.0 mL or less, use Table 6.

11.2.1 Selection of Tubes

If more than three rows of tubes are inoculated with sample (e.g., volumes/dilutions), select the most appropriate rows of tubes according to the criteria provided below. Examples of row selections and MPN/100 mL values are provided in Table 4.

11.2.1.1

Choose the smallest volume or the highest dilution giving positive results in all five tubes inoculated plus the two succeeding lower concentrations. In Example A from Table 4, 10 mL is a smaller volume than 20 mL and is the lowest volume giving positive results in all five tubes.

11.2.1.2

If the largest volume tested has less than five tubes with positive results, select it and the next two volumes (Table 4, Examples B and C).

11.2.1.3

When a positive result occurs in a smaller volume than the three rows selected according to the rules above, change the selection to the largest volume that has less than five positive results and the next two smaller volumes (Table 4, Example D).

11.2.1.4

When the selection rules above have left unselected any smaller volumes with positive results, add those positive tubes to the row of tubes for the smallest volume selected (Table 4, Example E).

11.2.1.5

If there were not enough lower volumes analyzed to select three dilutions using the rules above, then select the three smallest volumes (Table 4, Example F).

Table 4. Examples of Appropriate Tube Selection and MPN/100 mL[1]

Example	20 mL	10 mL	1.0 mL	0.1 mL	Significant Dilutions	Table	MPN Index	MPN/100 mL
A	5/5	5/5	3/5	0/5	5-3-0	6	0.792	79.2
B	4/5	5/5	1/5	0/5	4-5-1	5	0.1524	15.24
C	0/5	1/5	0/5	0/5	0-1-0	5	0.0067	0.67
D	5/5	3/5	1/5	1/5	3-1-1	6	0.137	13.7
E	4/5	4/5	0/5	1/5	4-4-1	5	0.1181	11.81
F	5/5	5/5	5/5	2/5	5-5-2	6	5.422	542.2

[1] Appropriate volumes are underlined and the largest sample volumes analyzed are highlighted.

11.2.2

For calculation of MPN/100 mL when additional dilutions are analyzed (e.g., 10^{-2}, 10^{-3}), obtain the MPN index value from Table 6 using the number of positive tubes in the three selected dilutions. Calculate MPN/100 mL using the equation below.

$$\text{MPN/100 mL} = \frac{\text{MPN Index from Table 6}}{\text{Middle volume analyzed in the series used for MPN determination}} \times 100$$

For example, a dilution series of 10^{-3}, 10^{-4}, 10^{-5}, with the following positive tubes 5, 1, 0, respectively, would be:

$$\text{MPN/100 mL} = \frac{0.329}{10^{-4}} \times 100 = 3.29 \times 10^{5}$$

Table 5. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used and Sample Inoculation Volumes are 20.0, 10.0, and 1.0 mL [1]

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.006473	----	0.0223	1-3-0	0.0312	0.0055	0.0678
0-0-1	0.0065	0.0012	0.0223	1-3-1	0.0393	0.0092	0.0821
0-0-2	0.0130	0.0012	0.0352	1-3-2	0.0475	0.0132	0.0967
0-0-3	0.0195	0.0012	0.0472	1-3-3	0.0559	0.0173	0.1119
0-0-4	0.0262	0.0033	0.0589	1-3-4	0.0644	0.0216	0.1277
0-0-5	0.0328	0.0062	0.0706	1-3-5	0.0730	0.0260	0.1444
0-1-0	0.0067	0.0012	0.0228	1-4-0	0.0409	0.0099	0.0849
0-1-1	0.0134	0.0012	0.0360	1-4-1	0.0495	0.0141	0.1002
0-1-2	0.0202	0.0012	0.0483	1-4-2	0.0583	0.0185	0.1163
0-1-3	0.0270	0.0037	0.0604	1-4-3	0.0672	0.0231	0.1331
0-1-4	0.0339	0.0067	0.0725	1-4-4	0.0763	0.0277	0.1509
0-1-5	0.0408	0.0099	0.0847	1-4-5	0.0855	0.0324	0.1700
0-2-0	0.0138	0.0012	0.0367	1-5-0	0.0517	0.0152	0.1042
0-2-1	0.0208	0.0012	0.0495	1-5-1	0.0609	0.0199	0.1212
0-2-2	0.0279	0.0040	0.0619	1-5-2	0.0703	0.0247	0.1391
0-2-3	0.0350	0.0072	0.0745	1-5-3	0.0799	0.0296	0.1583
0-2-4	0.0422	0.0106	0.0871	1-5-4	0.0897	0.0346	0.1790
0-2-5	0.0494	0.0141	0.1001	1-5-5	0.0998	0.0397	0.2015
0-3-0	0.0215	0.0012	0.0507	2-0-0	0.0155	0.0012	0.0404
0-3-1	0.0288	0.0044	0.0636	2-0-1	0.0226	0.0018	0.0526
0-3-2	0.0362	0.0077	0.0766	2-0-2	0.0303	0.0051	0.0662
0-3-3	0.0437	0.0113	0.0898	2-0-3	0.0382	0.0087	0.0801
0-3-4	0.0512	0.0051	0.1243	2-0-4	0.0462	0.0125	0.0943
0-3-5	0.0588	0.0095	0.1428	2-0-5	0.0543	0.0165	0.1090
0-4-0	0.0299	0.0049	0.0654	2-1-0	0.0234	0.0022	0.0540
0-4-1	0.0375	0.0084	0.0789	2-1-1	0.0315	0.0056	0.0683
0-4-2	0.0453	0.0121	0.0927	2-1-2	0.0397	0.0094	0.0827
0-4-3	0.0531	0.0160	0.1069	2-1-3	0.0480	0.0134	0.0976
0-4-4	0.0611	0.0200	0.1216	2-1-4	0.0565	0.0177	0.1131
0-4-5	0.0691	0.0241	0.1369	2-1-5	0.0652	0.0221	0.1293
0-5-0	0.0390	0.0090	0.0814	2-2-0	0.0327	0.0062	0.0705
0-5-1	0.0470	0.0129	0.0958	2-2-1	0.0413	0.0101	0.0856
0-5-2	0.0553	0.0170	0.1107	2-2-2	0.0501	0.0144	0.1013
0-5-3	0.0636	0.0212	0.1262	2-2-3	0.0590	0.0189	0.1176
0-5-4	0.0720	0.0255	0.1425	2-2-4	0.0681	0.0236	0.1349
0-5-5	0.0806	0.0299	0.1596	2-2-5	0.0774	0.0283	0.1533
1-0-0	0.0072	0.0012	0.0241	2-3-0	0.0431	0.0110	0.0887
1-0-1	0.0139	0.0012	0.0369	2-3-1	0.0523	0.0155	0.1053
1-0-2	0.0209	0.0012	0.0497	2-3-2	0.0617	0.0203	0.1227
1-0-3	0.0281	0.0041	0.0623	2-3-3	0.0714	0.0252	0.1412
1-0-4	0.0353	0.0073	0.0749	2-3-4	0.0813	0.0303	0.1611
1-0-5	0.0425	0.0107	0.0878	2-3-5	0.0914	0.0354	0.1826
1-1-0	0.0144	0.0012	0.0377	2-4-0	0.0547	0.0168	0.1098
1-1-1	0.0217	0.0013	0.0509	2-4-1	0.0647	0.0218	0.1284
1-1-2	0.0290	0.0045	0.0640	2-4-2	0.0750	0.0271	0.1484
1-1-3	0.0365	0.0079	0.0771	2-4-3	0.0855	0.0325	0.1700
1-1-4	0.0441	0.0115	0.0905	2-4-4	0.0964	0.0380	0.1937
1-1-5	0.0517	0.0153	0.1043	2-4-5	0.1076	0.0436	0.2201
1-2-0	0.0224	0.0017	0.0523	2-5-0	0.0681	0.0235	0.1349
1-2-1	0.0301	0.0050	0.0658	2-5-1	0.0791	0.0292	0.1566
1-2-2	0.0379	0.0085	0.0795	2-5-2	0.0904	0.0349	0.1805
1-2-3	0.0457	0.0123	0.0935	2-5-3	0.1021	0.0409	0.2070
1-2-4	0.0537	0.0162	0.1079	2-5-4	0.1143	0.0469	0.2372
1-2-5	0.0618	0.0203	0.1229	2-5-5	0.1268	0.0531	0.2725

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
3-0-0	0.0255	0.0028	0.0585	4-3-0	0.0797	0.0295	0.1579
3-0-1	0.0330	0.0063	0.0710	4-3-1	0.0937	0.0366	0.1877
3-0-2	0.0417	0.0103	0.0863	4-3-2	0.1086	0.0441	0.2228
3-0-3	0.0506	0.0147	0.1023	4-3-3	0.1245	0.0520	0.2656
3-0-4	0.0598	0.0193	0.1191	4-3-4	0.1414	0.0602	0.3218
3-0-5	0.0691	0.0241	0.1368	4-3-5	0.1595	0.0686	0.4067
3-1-0	0.0344	0.0069	0.0734	4-4-0	0.1012	0.0404	0.2049
3-1-1	0.0435	0.0112	0.0896	4-4-1	0.1181	0.0489	0.2476
3-1-2	0.0529	0.0159	0.1065	4-4-2	0.1364	0.0578	0.3038
3-1-3	0.0626	0.0207	0.1244	4-4-3	0.1563	0.0672	0.3890
3-1-4	0.0725	0.0258	0.1434	4-4-4	0.1780	0.0770	0.5273
3-1-5	0.0827	0.0310	0.1640	4-4-5	0.2015	0.0873	0.6411
3-2-0	0.0456	0.0122	0.0932	4-5-0	0.1304	0.0549	0.2836
3-2-1	0.0555	0.0171	0.1112	4-5-1	0.1524	0.0653	0.3687
3-2-2	0.0657	0.0223	0.1303	4-5-2	0.1769	0.0766	0.5210
3-2-3	0.0763	0.0277	0.1510	4-5-3	0.2046	0.0886	0.6528
3-2-4	0.0872	0.0333	0.1735	4-5-4	0.2357	0.1015	0.7516
3-2-5	0.0984	0.0390	0.1984	4-5-5	0.2708	0.1150	0.8426
3-3-0	0.0583	0.0186	0.1164	5-0-0	0.0549	0.0162	0.1116
3-3-1	0.0693	0.0241	0.1371	5-0-1	0.0637	0.0213	0.1265
3-3-2	0.0806	0.0299	0.1597	5-0-2	0.0763	0.0277	0.1510
3-3-3	0.0924	0.0359	0.1847	5-0-3	0.0896	0.0345	0.1787
3-3-4	0.1046	0.0421	0.2128	5-0-4	0.1037	0.0417	0.2107
3-3-5	0.1173	0.0484	0.2452	5-0-5	0.0953	0.0165	0.2234
3-4-0	0.0733	0.0262	0.1450	5-1-0	0.0678	0.0234	0.1344
3-4-1	0.0856	0.0325	0.1700	5-1-1	0.0816	0.0304	0.1618
3-4-2	0.0984	0.0390	0.1982	5-1-2	0.0963	0.0379	0.1936
3-4-3	0.1118	0.0457	0.2307	5-1-3	0.1121	0.0459	0.2316
3-4-4	0.1258	0.0526	0.2695	5-1-4	0.1291	0.0542	0.2796
3-4-5	0.1405	0.0597	0.3184	5-1-5	0.1293	0.0304	0.3090
3-5-0	0.0913	0.0354	0.1825	5-2-0	0.0879	0.0337	0.1751
3-5-1	0.1055	0.0426	0.2150	5-2-1	0.1046	0.0421	0.2128
3-5-2	0.1204	0.0500	0.2538	5-2-2	0.1227	0.0511	0.2605
3-5-3	0.1362	0.0577	0.3029	5-2-3	0.1427	0.0608	0.3267
3-5-4	0.1529	0.0656	0.3715	5-2-4	0.1646	0.0710	0.4385
3-5-5	0.1707	0.0738	0.4795	5-2-5	0.1767	0.0503	0.5230
4-0-0	0.0381	0.0082	0.0809	5-3-0	0.1151	0.0474	0.2394
4-0-1	0.0461	0.0125	0.0942	5-3-1	0.1368	0.0580	0.3050
4-0-2	0.0563	0.0175	0.1126	5-3-2	0.1614	0.0695	0.4183
4-0-3	0.0668	0.0229	0.1323	5-3-3	0.1895	0.0821	0.5899
4-0-4	0.0777	0.0284	0.1537	5-3-4	0.2216	0.0957	0.7101
4-0-5	0.0890	0.0342	0.1773	5-3-5	0.2527	0.0814	0.7971
4-1-0	0.0484	0.0136	0.0983	5-4-0	0.1571	0.0676	0.3935
4-1-1	0.0592	0.0190	0.1181	5-4-1	0.1907	0.0826	0.5954
4-1-2	0.0705	0.0248	0.1395	5-4-2	0.2319	0.0999	0.7409
4-1-3	0.0822	0.0308	0.1631	5-4-3	0.2834	0.1196	0.8726
4-1-4	0.0945	0.0370	0.1894	5-4-4	0.3475	0.1417	1.0160
4-1-5	0.1072	0.0434	0.2193	5-4-5	0.4256	0.1437	1.1800
4-2-0	0.0626	0.0207	0.1244	5-5-0	0.2398	0.0762	0.7629
4-2-1	0.0748	0.0269	0.1479	5-5-1	0.3477	0.1172	1.0160
4-2-2	0.0875	0.0335	0.1742	5-5-2	0.5422	0.1791	1.4190
4-2-3	0.1009	0.0403	0.2041	5-5-3	0.9178	0.2672	2.2010
4-2-4	0.1150	0.0473	0.2392	5-5-4	1.6090	0.3837	4.1030
4-2-5	0.1299	0.0546	0.2820	5-5-5	>1.6090	0.3837	----

(1) Table was developed using the MPN calculator developed by Albert Klee (Reference 15.10).

Table 6. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used and Sample Inoculation Volumes are 10.0, 1.0, and 0.1 mL [1]

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.018	---	0.063	1-3-0	0.083	0.012	0.196
0-0-1	0.018	0.003	0.063	1-3-1	0.104	0.020	0.243
0-0-2	0.036	0.003	0.101	1-3-2	0.125	0.029	0.296
0-0-3	0.054	0.003	0.137	1-3-3	0.147	0.038	0.364
0-0-4	0.072	0.008	0.174	1-3-4	0.169	0.048	0.460
0-0-5	0.091	0.015	0.212	1-3-5	0.191	0.057	0.566
0-1-0	0.018	0.003	0.063	1-4-0	0.105	0.021	0.245
0-1-1	0.036	0.003	0.101	1-4-1	0.127	0.030	0.300
0-1-2	0.055	0.003	0.138	1-4-2	0.148	0.039	0.370
0-1-3	0.073	0.008	0.175	1-4-3	0.170	0.048	0.468
0-1-4	0.091	0.015	0.214	1-4-4	0.193	0.058	0.575
0-1-5	0.110	0.023	0.256	1-4-5	0.215	0.067	0.657
0-2-0	0.037	0.003	0.102	1-5-0	0.128	0.030	0.303
0-2-1	0.055	0.003	0.139	1-5-1	0.150	0.040	0.375
0-2-2	0.074	0.008	0.176	1-5-2	0.172	0.049	0.477
0-2-3	0.092	0.015	0.215	1-5-3	0.195	0.058	0.583
0-2-4	0.111	0.023	0.258	1-5-4	0.217	0.068	0.664
0-2-5	0.129	0.031	0.307	1-5-5	0.240	0.077	0.731
0-3-0	0.056	0.003	0.140	2-0-0	0.045	0.003	0.119
0-3-1	0.074	0.009	0.177	2-0-1	0.068	0.006	0.164
0-3-2	0.093	0.016	0.217	2-0-2	0.091	0.015	0.213
0-3-3	0.112	0.023	0.260	2-0-3	0.115	0.025	0.269
0-3-4	0.130	0.031	0.310	2-0-4	0.139	0.035	0.338
0-3-5	0.149	0.039	0.372	2-0-5	0.164	0.046	0.437
0-4-0	0.075	0.009	0.179	2-1-0	0.068	0.006	0.166
0-4-1	0.094	0.016	0.219	2-1-1	0.092	0.015	0.216
0-4-2	0.112	0.024	0.263	2-1-2	0.116	0.025	0.272
0-4-3	0.131	0.032	0.313	2-1-3	0.141	0.036	0.343
0-4-4	0.150	0.040	0.377	2-1-4	0.166	0.046	0.447
0-4-5	0.169	0.048	0.462	2-1-5	0.192	0.057	0.571
0-5-0	0.094	0.016	0.221	2-2-0	0.093	0.016	0.218
0-5-1	0.113	0.024	0.265	2-2-1	0.118	0.026	0.276
0-5-2	0.133	0.032	0.317	2-2-2	0.143	0.036	0.349
0-5-3	0.152	0.040	0.382	2-2-3	0.168	0.047	0.456
0-5-4	0.171	0.048	0.470	2-2-4	0.194	0.058	0.581
0-5-5	0.190	0.056	0.563	2-2-5	0.221	0.069	0.675
1-0-0	0.020	0.003	0.068	2-3-0	0.119	0.026	0.279
1-0-1	0.040	0.003	0.108	2-3-1	0.144	0.037	0.355
1-0-2	0.060	0.003	0.149	2-3-2	0.170	0.048	0.467
1-0-3	0.081	0.011	0.191	2-3-3	0.197	0.059	0.591
1-0-4	0.101	0.019	0.236	2-3-4	0.223	0.070	0.683
1-0-5	0.122	0.028	0.287	2-3-5	0.251	0.082	0.759
1-1-0	0.040	0.003	0.109	2-4-0	0.146	0.038	0.361
1-1-1	0.061	0.003	0.150	2-4-1	0.172	0.049	0.477
1-1-2	0.081	0.011	0.192	2-4-2	0.199	0.060	0.600
1-1-3	0.102	0.019	0.238	2-4-3	0.226	0.072	0.692
1-1-4	0.123	0.028	0.290	2-4-4	0.254	0.083	0.768
1-1-5	0.144	0.037	0.354	2-4-5	0.282	0.094	0.836
1-2-0	0.061	0.003	0.151	2-5-0	0.174	0.050	0.488
1-2-1	0.082	0.012	0.194	2-5-1	0.201	0.061	0.610
1-2-2	0.103	0.020	0.240	2-5-2	0.229	0.073	0.700
1-2-3	0.124	0.029	0.293	2-5-3	0.257	0.084	0.776
1-2-4	0.146	0.038	0.359	2-5-4	0.286	0.095	0.845
1-2-5	0.167	0.047	0.451	2-5-5	0.315	0.107	0.910

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
3-0-0	0.079	0.010	0.188	4-3-0	0.271	0.090	0.809
3-0-1	0.106	0.021	0.246	4-3-1	0.326	0.111	0.934
3-0-2	0.135	0.033	0.323	4-3-2	0.386	0.132	1.060
3-0-3	0.165	0.046	0.440	4-3-3	0.451	0.154	1.192
3-0-4	0.196	0.059	0.589	4-3-4	0.521	0.176	1.331
3-0-5	0.229	0.073	0.699	4-3-5	0.593	0.196	1.477
3-1-0	0.107	0.022	0.250	4-4-0	0.335	0.114	0.953
3-1-1	0.137	0.034	0.329	4-4-1	0.398	0.137	1.084
3-1-2	0.167	0.047	0.452	4-4-2	0.466	0.159	1.223
3-1-3	0.199	0.060	0.601	4-4-3	0.539	0.181	1.368
3-1-4	0.232	0.074	0.710	4-4-4	0.615	0.202	1.521
3-1-5	0.267	0.088	0.800	4-4-5	0.693	0.223	1.681
3-2-0	0.138	0.035	0.335	4-5-0	0.411	0.141	1.111
3-2-1	0.170	0.048	0.464	4-5-1	0.483	0.164	1.256
3-2-2	0.202	0.062	0.613	4-5-2	0.559	0.187	1.409
3-2-3	0.236	0.076	0.720	4-5-3	0.639	0.209	1.570
3-2-4	0.271	0.090	0.810	4-5-4	0.722	0.230	1.739
3-2-5	0.308	0.104	0.894	4-5-5	0.806	0.250	1.916
3-3-0	0.172	0.049	0.477	5-0-0	0.240	0.076	0.763
3-3-1	0.205	0.063	0.624	5-0-1	0.314	0.106	0.908
3-3-2	0.240	0.077	0.731	5-0-2	0.427	0.146	1.142
3-3-3	0.276	0.092	0.821	5-0-3	0.578	0.192	1.446
3-3-4	0.313	0.106	0.906	5-0-4	0.759	0.239	1.816
3-3-5	0.352	0.120	0.989	5-0-5	0.953	0.165	2.234
3-4-0	0.209	0.064	0.636	5-1-0	0.329	0.112	0.940
3-4-1	0.244	0.079	0.742	5-1-1	0.456	0.156	1.202
3-4-2	0.281	0.093	0.833	5-1-2	0.631	0.207	1.553
3-4-3	0.319	0.108	0.918	5-1-3	0.839	0.257	1.985
3-4-4	0.358	0.123	1.002	5-1-4	1.062	0.304	2.485
3-4-5	0.399	0.137	1.086	5-1-5	1.293	0.304	3.090
3-5-0	0.248	0.080	0.753	5-2-0	0.493	0.167	1.276
3-5-1	0.286	0.095	0.844	5-2-1	0.700	0.224	1.694
3-5-2	0.325	0.110	0.931	5-2-2	0.944	0.280	2.213
3-5-3	0.365	0.125	1.017	5-2-3	1.205	0.331	2.843
3-5-4	0.407	0.140	1.103	5-2-4	1.479	0.381	3.714
3-5-5	0.450	0.154	1.189	5-2-5	1.767	0.503	5.230
4-0-0	0.130	0.031	0.311	5-3-0	0.792	0.247	1.886
4-0-1	0.166	0.046	0.445	5-3-1	1.086	0.308	2.544
4-0-2	0.207	0.064	0.631	5-3-2	1.406	0.368	3.445
4-0-3	0.253	0.082	0.764	5-3-3	1.750	0.434	5.131
4-0-4	0.302	0.102	0.881	5-3-4	2.122	0.529	6.798
4-0-5	0.355	0.121	0.996	5-3-5	2.527	0.814	7.971
4-1-0	0.169	0.048	0.460	5-4-0	1.299	0.348	3.108
4-1-1	0.212	0.066	0.646	5-4-1	1.724	0.429	4.975
4-1-2	0.258	0.085	0.779	5-4-2	2.212	0.563	7.087
4-1-3	0.310	0.105	0.898	5-4-3	2.781	0.882	8.600
4-1-4	0.365	0.125	1.016	5-4-4	3.454	1.159	10.110
4-1-5	0.425	0.145	1.138	5-4-5	4.256	1.437	11.800
4-2-0	0.216	0.067	0.661	5-5-0	2.398	0.762	7.629
4-2-1	0.264	0.087	0.794	5-5-1	3.477	1.172	10.160
4-2-2	0.317	0.108	0.915	5-5-2	5.422	1.791	14.190
4-2-3	0.375	0.129	1.037	5-5-3	9.178	2.672	22.010
4-2-4	0.438	0.150	1.164	5-5-4	16.090	3.837	41.030
4-2-5	0.504	0.171	1.297	5-5-5	>16.090	3.837	-----

(1) Table was developed using the MPN calculator developed by Albert Klee (Reference 15.10).

12.0
Protocol Performance

Culture-based procedures were evaluated for *S.* Typhi in a reference matrix (PBS) and two matrices of interest (drinking water and surface water) during a single-laboratory verification study. Results for surface water analyses were unacceptable; therefore, surface water procedures were not included in this SAP. Details regarding procedure performance are provided in the study report (Reference 15.11). Verification results are provided in Table 7.

Table 7. ***Salmonella*** Typhi Results for PBS, Drinking Water, and Surface Water Verification Analyses [1]

Date	Sample	Spike Level (CFU/100 mL)	Medium	MPN Combo	*S.* Typhi (MPN/100 mL)	Recovery (%)	Mean Recovery (%)	SD (%)	RSD (%)
PBS Samples									
12/16/08	Unspiked	NA	NA	0-0-0	<1.08				
1/09/09		NA	NA	0-0-0	<1.08				
12/16/08	Spiked	32	BS_D [2]	2-3-1	12.88	37.00	71.18	29.16	40.97
				2-3-3	19.33	57.00			
1/09/09		24		3-3-0	23.98	95.41			
				3-3-0	23.98	95.41			
12/16/08	Spiked	32	MM_D [2]	2-3-1	12.88	37.00	94.35	67.04	71.06
				2-3-3	19.33	57.00			
1/09/09		24		3-3-1	46.22	188.08			
				3-3-0	23.98	95.41			
Drinking Water Samples									
12/16/08	Unspiked	NA	NA	0-0-0	<1.08				
				0-0-0	<1.08				
1/9/09	Unspiked	NA	NA	0-0-0	<1.08				
				0-0-0	<1.08				
12/16/08	Spiked	32	BS_D [2]	3-3-2	109.9	340.00	307.24 [3]	150.99	49.14
				3-3-2	109.9	340.00			
1/9/09		24		3-3-0	23.98	95.41			
				3-3-2	109.9	453.41			
12/16/08	Spiked	32	MM_D [2]	3-3-2	109.9	340.00	307.24	150.99	49.14
				3-3-2	109.9	340.00			
1/9/09		24		3-3-0	23.98	95.41			
				3-3-2	109.9	453.41			

Date	Sample	Spike Level (CFU/100 mL)	Medium	MPN Combo	*S.* Typhi (MPN/100 mL)	Recovery (%)	Mean Recovery (%)	SD (%)	RSD (%)
Surface Water Samples									
12/16/08	Unspiked	NA	NA	0-0-0	<1.08				
				0-0-0	<1.08				
1/9/09	Unspiked	NA	NA	0-0-0	<1.08				
				0-0-0	<1.08				
12/16/08	Spiked	32	BS_D [2]	0-0-0	<1.08	0.00	0.00	0.00	NA
				0-0-0	<1.08	0.00			
1/9/09		24		0-0-0	<1.08	0.00			
				0-0-0	<1.08	0.00			
12/16/08	Spiked	32	MM_D [2]	0-0-0	<1.08	0.00	0.00	0.00	NA
				0-0-0	<1.08	0.00			
1/9/09		24		0-0-0	<1.08	0.00			
				0-0-0	<1.08	0.00			

[1] These values are based on a 9-tube, as opposed to a 15-tube, MPN
[2] Supplemented with 2,3-dihydroxybenzoate
[3] It should be noted that while 307% recovery may seem high, the variability in the 109.9 MPN value ranges from 22.5 – 307.5 for the 95% confidence internal.

BS – Bismuth sulfite
CFU/100mL – Colony forming unit per 100 milliliter
MM – Miller-Mallinson
NA – Not applicable
RSD – Relative standard deviation
SCB – Selenite cystine broth
SD – Standard deviation

13.0
Pollution Prevention

13.1
The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

13.2
Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

14.0
Waste Management

14.1
It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

14.2
Samples, reference materials, and equipment known or suspected to be contaminated with viable *S.* Typhi must be sterilized prior to disposal.

14.3
For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* (Reference 15.12) and *Less Is Better: Laboratory Chemical Management for Waste Reduction* (Reference 15.13), both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

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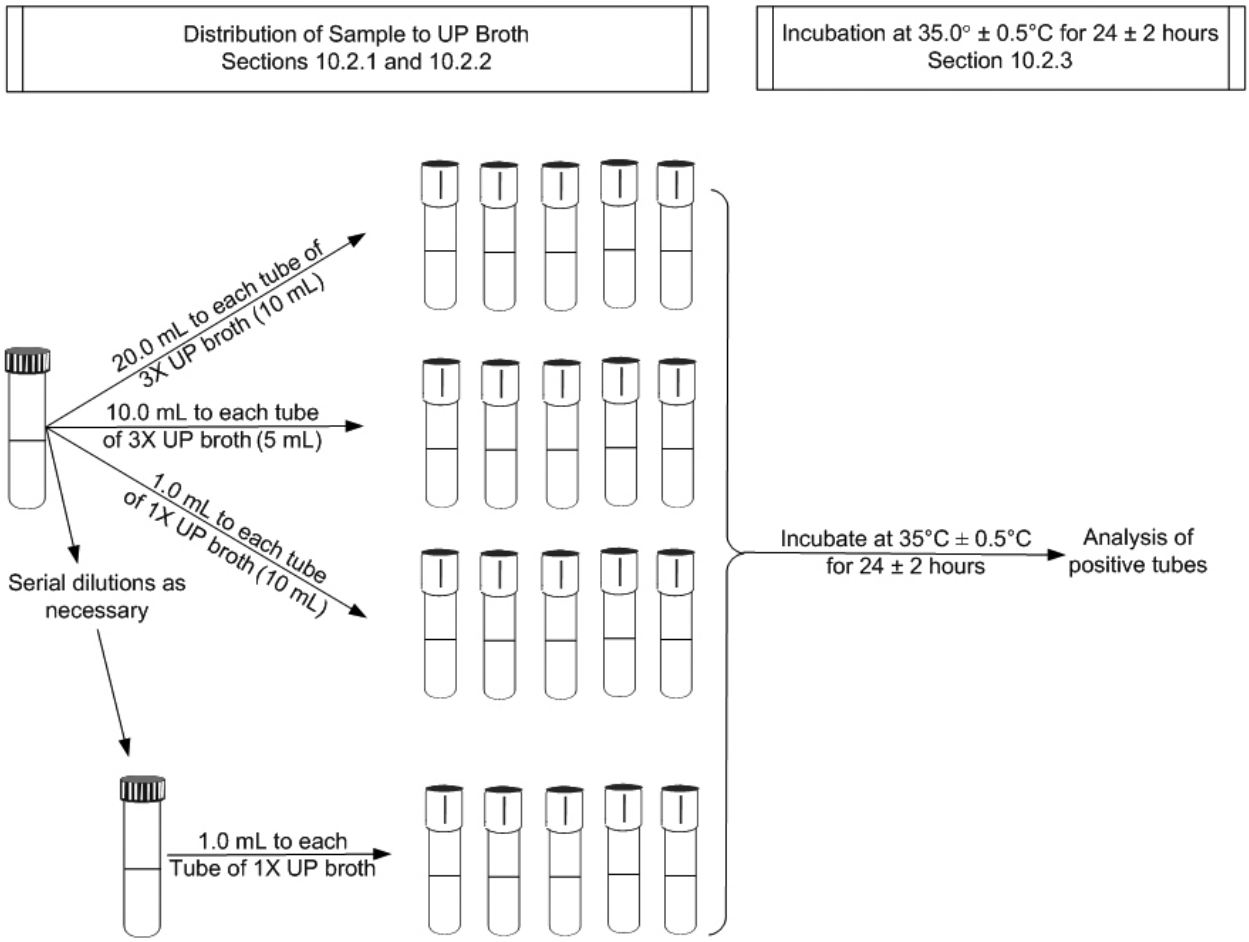
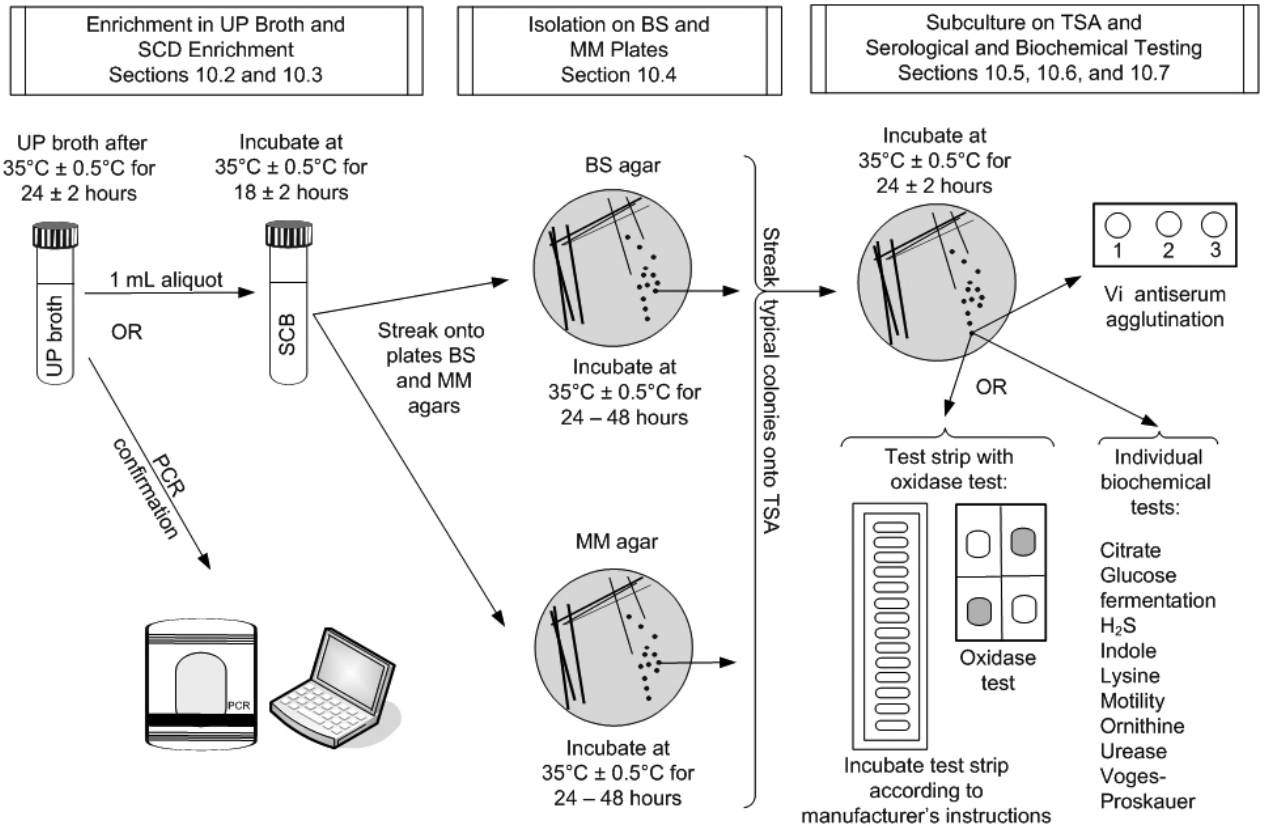
16.0
Flowcharts and Diagrams

16.1 Quantitative Analysis Dilution Scheme



16.2 Identification Flowchart



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